



Lack of evidence for post-vaccine onset of autoimmune/lymphoproliferative disorders, during a nine-month follow-up in multiply vaccinated Italian military personnel



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ABSTRACT

Anecdotal case reports, amplified by mass media and internet-based opinion groups, have recently indicated vaccinations as possibly responsible for autoimmunity/lymphoproliferation development. Multiply vaccinated Italian military personnel (group 1, operating in Italy, group 2, operating in Lebanon) were followed-up for nine months to monitor possible post-vaccine autoimmunity/lymphoproliferation onset. No serious adverse event was noticed in both groups. Multivariate analysis of intergroup differences only showed a significant association between lymphocyte increase and tetanus/diphtheria vaccine administration. A significant post-vaccine decrease in autoantibody positivity was observed. Autoantibodies were also studied by microarray analysis of self-proteins in subjects exposed to ≥ 4 concurrent vaccinations, without observing significant difference among baseline and one and nine months post-vaccine. Moreover, HLA-A2 subjects have been analyzed for the possible CD8T-cell response to apoptotic self-epitopes, without observing significant difference between baseline and one month post-vaccine. Multiple vaccinations in young adults are safe and not associated to autoimmunity/lymphoproliferation onset during a nine-month-long follow-up.

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Abbreviations: BCG, Bacillus Calmette–Guérin; CDC, Centers for Disease Control and Prevention; Td, tetanus/diphtheria; MMR, measles/mumps/rubella; HA, hepatitis A; HB, hepatitis B; T0, time 0; T1, time 1; T2, time 2; Ig, immunoglobulins; ANA, antinuclear antibodies; anti-dsDNA, anti-double-stranded DNA antibodies; anti-ENAs, anti-extractable nuclear antigens antibodies; APL, anti-phospholipid antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; RF, rheumatoid factor; HLA, Human leukocyte antigen; PCR-RDB, PCR and Reverse Dot Blot; commercial kit LIPA, -; PCR-SSP, DNA amplification using sequence specific primer; MFI, mean fluorescence intensity; IFN, interferon; IFI, indirect immunofluorescence; ELISA, enzyme-linked-immunosorbent assay; INGM, National Institute for Molecular Genetics; HSA, human serum albumin; Elispot, enzyme linked immunospot; ACTB, actin cytoplasmic 1; ROK, heterogeneous nuclear ribonucleoprotein; LAM1, lamin B1; MYH9, non-muscle myosin heavy chain 9; GDIS, rho GDP dissociation inhibitor 2; VIME, vimentin; PSA1, proteasome component C2; RLA, 60S acidic ribosomal protein P2; SFC, spot forming cells.

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1. Introduction

The armed forces of all countries in the world protect their personnel against the risk of infections by prophylactic vaccination programs in order to maintain operational readiness [1]. In fact, the military are particularly vulnerable to infectious diseases as a consequence of community life, often in precarious hygienic and hostile climatic conditions, and rapid relocations to areas with different epidemiology of infectious diseases for operational missions [2]. Despite the undeniable merits of vaccinations in the control of infectious diseases [3] and the substantial vaccine safety [4], recently, especially in developed countries, mass media have dedicated special attention to the potential vaccine-induced damages. The emergence of some cases of Hodgkin's and non-Hodgkin's lymphomas in otherwise healthy military personnel of the Italian armed forces has fueled the argument that vaccinations, specifically multiple vaccinations, may represent important co-factors for the induction of lymphoproliferative disorders. This climate of opinion has probably contributed to the judgment that ascribed the death of an enlisted military for non-Hodgkin's lymphoma to the mandatory multiple vaccination schedule administered to the soldier during his military service [5]. A careful analysis of nearly 1800 papers selected on PubMed according to the key words "lymphoproliferative disorders and vaccinations" retrieved only few anecdotal, not confirmed, reports of increased lymphoproliferation risk following influenza [6–7] and tuberculosis [Bacillus Calmette-Guérin (BCG)] [8–9] vaccines, in addition to the description of cutaneous pseudo-lymphoma by hepatitis B and A vaccines [10]. Conversely, influenza and BCG vaccinations have even been associated with a protective effect against lymphomas [11–12]. The significant increase of monoclonal gammopathies observed in individuals who had received multiple, even experimental, vaccinations decades before in comparison to controls is quite inexplicable. In fact, such an increase was not correlated with the type or amount of received vaccines, nor was it present on the occasion of the last check, thirty years before and twenty-five years after the end of vaccine stimulation [13]. More recently, vaccine adjuvants have been suspected as possible inducer of autoimmunity and lymphoma, as a presumed consequence of chronic inflammation due to immune system stimulation [14–15]. Moreover, a vaccine/adjuvant-driven evolution to lymphoproliferative disorders has even been observed in animals [16–20]. In healthy people, vaccine administration has occasionally been related to the occurrence of side effects, as reported in the probably most susceptible population of premature newborns [21] or in adults as kidney pathology rarely observed soon after vaccination with H1N1 influenza vaccine [22] and of autoimmune diseases, such as Guillain-Barré syndrome, immune thrombocytopenic purpura and myopericarditis, after swine flu (1976), measles-mumps-rubella and smallpox vaccinations, respectively [23,24]. Actually, in genetically predisposed subjects, the vaccination may rarely trigger an autoimmune reaction, through the mechanisms of molecular mimicry, bystander activation [24,25], epitope spreading or polyclonal activation [15].

The Centers for Disease Control and Prevention in 1998 have identified some pathological forms exclusive of the military returned from war campaigns in some war scenarios, such as the Multi-Symptomatic Syndrome, defined by fatigue and post-traumatic stress reaction [26]. In veterans of the first Gulf War the Multi-Symptomatic Syndrome has been linked to immune dysfunction, characterized by Th1 activation and IL10 secretion by memory cells [27]. Actually, some authors have suggested that immunological (concurrent vaccinations), accompanied by emotional (the mission to war theater) stress, could have contributed to cause an immunological imbalance, able to induce a state of ill health in veterans of the Gulf War [28,29]. This hypothesis has not been confirmed and more recently has been downgraded by the same research group [30]. Moreover, in the case of anthrax vaccination, Mahan et al. showed significant differences, in terms of health status, between the group of subjects who did report having received anthrax vaccination and those who did not. However, when the authors

compared the group who self-reported and the veterans for whom vaccination records were available, these differences disappeared [31].

This background considered, the current study, based on clinical, molecular, and immunological analysis of two military groups, one operating in Italy and the other in Lebanon, was designed as an active surveillance for possible post-vaccine onset of clinical and/or laboratory autoimmunity/lymphoproliferation during a nine-month-long period.

2. Materials and methods

2.1. Study population

From September 2012 to June 2014 two groups of Italian military personnel, the first represented by newly-recruited students of military schools residing in Italy for at least 3 years and the other by soldiers operating abroad (Lebanon) for nine months, were enrolled. Exclusion criteria were: pregnancy, immunodepression, vaccine hypersensitivity.

The study was approved by the Italian Ministry of Defense ethical committee in July 2011, and registered in clinicaltrials.gov in 2012 with the identifier NCT01807780.

2.2. Vaccination schedule

At enrollment, informed consent and medical history of all individuals were collected. The vaccination schedule was personalized on the basis of information on history of infectious diseases and already received vaccinations; moreover, the vaccination schedule was even tailored on type of employment, either national or abroad. Administered vaccines were the following: tetanus/diphtheria (Td, DifTetAll-Novartis Vaccines and Diagnostics, Siena, Italy), inactivated polio (Imovax polio-Sanofi Pasteur MSD SpA, Roma, Italy), measles/mumps/rubella (MMR, Priorix -GlaxoSmithKline SpA, Verona, Italy), chickenpox (Varivax-Sanofi Pasteur MSD SpA, Roma, Italy) polysaccharide tetravalent (A, C, W₁₃₅, Y) meningococcal meningitis (Mencevax-PziferSrl, Latina, Italy), hepatitis A (HA, Epaxal-Crucell Italy Srl, Baranzate, Italy), hepatitis B (HB, Engerix B-GlaxoSmithKline SpA, Verona, Italy), influenza (Fluad-Seqirus Srl, Siena, Italy), and typhoid (Vivotif Berna-PaxVax Ltd., Birmingham, UK). Vaccines were generally administered the same day (in different arms), but in a few cases up to two weeks apart. However, a considerably lower number of subjects than expected were immunized with the aforementioned vaccines, as a consequence of previous vaccination records and history of infectious diseases.

2.3. Safety

Blood samples were collected before (time 0 [T0]) and one and nine months after vaccination (time 1 [T1] and time 2 [T2]) in group 1; at T0 and T2, following mission to Lebanon, in group 2.

Vaccine safety was examined by active monitoring of adverse events (at vaccination each subject was provided with a card to record any possible adverse events [local or systemic reaction, mild or severe]) and by the evaluation of the peripheral blood cell count, serum protein electrophoresis and serum immunoglobulins (Ig), to monitor the possible onset of signs suggestive of lymphoproliferative disorders. Moreover, the search for autoantibodies (anti-nuclear [ANA], anti-double-stranded DNA [anti-dsDNA], anti-extractable nuclear antigens [anti-ENAs], anti-phospholipid [APL], anti-neutrophil cytoplasmic antibodies [ANCA] and rheumatoid factor [RF]), was performed.

In 12/172 (7%) group 1 subjects, who had received ≥ 4 vaccinations, the presence of self-antigens recognized by specific antibodies was studied by microarray analysis at T0, T1 and T2. In 29/172 (17%) group 1 subjects, including those studied by microarray analysis, the possible cellular response to apoptotic self-epitopes, as an equivalent of cellular autoimmunity, was even investigated [32].

2.4. Autoantibodies

ANA, anti-dsDNA and ANCA were detected by indirect immunofluorescence (IIF), using Hep-2 cells, *Crithidia luciliae* slides, formalin- and ethanol-fixed neutrophils as substrate, respectively. Titers $\geq 1:80$ (for ANA), $\geq 1:20$ (for anti-dsDNA) and $\geq 1:20$ (for ANCA) were considered positive. Positive samples were confirmed by enzyme-linked-immunosorbent assay (ELISA, commercial kit QUANTA Lite TM ANA-INOVA; QUANTA Lite dsDNA SC ELISA-INOVA; ELiA TM PR3S and ELiA TM MPOS - Phadia). Anti-ENAs were identified by ELISA (commercial kit ELISA QUANTA Lite TM-INOVA). Test results >20 IU/ml were considered positive. APL were analyzed by ELISA (commercial kit QUANTA Lite ACA IgM III, QUANTA Lite ACA IgG III, QUANTA Lite ACA IgA III-INOVA). Test results >20 MPL or >20 GPL or >20 APL were considered positive.

2.5. Microarray analysis

The high-density protein microarray used in this study included 1667 membrane-associated/secreted recombinant human proteins so far marginally characterized [33] and was generated in the National Institute for Molecular Genetics (INGM) laboratory [34]. Briefly, after expression in *E. coli* as His-GST-tagged fusions, the corresponding proteins were purified from the bacterial insoluble fraction (6 M urea) by affinity chromatography. As experimental positive controls, to assess the sensitivity and reproducibility of the array for data normalization, a curve of human IgG and the protein A from *S. aureus* were spotted on the slides, and negative controls, as human albumin (HSA), buffer, and water were also used. Each slide was incubated with individual sera (at a dilution of 1:300) and the sera reactivity was compared for the three time points. In order to detect the autoantibody-autoantigen recognition, the slides were probed with secondary antibody (Alexa-647-conjugated anti-human IgG) and the number of spots estimated with a mean fluorescence intensity (MFI) value.

Fluorescence signals were detected by using a ScanArray Gx PLUS (PerkinElmer, Bridgeport Avenue Shelton, USA) and processed using ImaGene 8.0 software (Biodiscovery Inc., CA, USA). Elaboration and analysis of image raw fluorescence intensity and normalization for each slide was performed on the basis of the signal distribution of the human Ig curves using INGM-developed software, as previously described [35]. Finally, the calculation of the frequency of recognition of each protein by each serum was performed.

2.6. Cellular response to apoptotic self-epitopes

In 29 group 1 subjects, selected on the basis of the number of received vaccinations and the expression of HLA-A2 allele, and in 15 age- and sex-matched HLA-A2 healthy controls not recently exposed to immunization stimuli, the autoreactive CD8⁺ T cell response to self-epitopes associated to apoptotic T cells (mostly derived from cytoskeletal proteins) was explored, according to the protocol of Rawson et al. [32]. Briefly, CD8⁺ T cells were purified, and functionally tested for their capacity to form interferon (IFN)- γ spots by enzyme-linked immunospot (Elispot), within 4–6 h of contact with a pool containing a total of 90 synthetic human leukocyte antigen (HLA)-A2-binding apoptotic self-epitopes (nonamer or decamer peptides) that had been selected for their capacity to bind the HLA-A2 molecule [32].

2.7. Synthetic peptides

Ninety synthetic peptides (nonamers or decamers) were prepared according to the sequence of the caspase-cleaved proteins (actin cytoplasmic 1 [ACTB], heterogeneous nuclear ribonucleoprotein [ROK], lamin B1 [LAM1], non-muscle myosin heavy chain 9 [MYH9], rho GDP dissociation inhibitor 2 [GDIS], vimentin [VIME], proteasome component C2 [PSA1], and 60S acidic ribosomal protein P2 [RLA]) that had been previously identified by the proteomic analyses of apoptotic T

cells, as described [32]. All the peptides (Supplementary Table 1) were selected for their capacity to bind the HLA-A2 molecule [32].

2.8. HLA typing

HLA class I typing was performed by PCR and Reverse Dot Blot (PCR-RDB) (commercial kit LIPA), and HLA class II DNA amplification using sequence specific primer (PCR-SSP) (commercial kit One-lambda), according to manufacturer's instructions.

2.9. Statistical analysis

Data were analyzed by a repeated measure ANOVA and Bonferroni's multiple comparison; in case of non-normal data distribution, analysis was performed after logarithmic transformation. Categorical variables were analyzed by Yates corrected, two tails, χ^2 test, whereas demographic data (mean age) by Student *t*-test. Values of $p \leq 0.05$ were considered significant. Statistical analysis was performed using the program package GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA). MFI signals were analyzed by Student *t*-test, using the TMEV software. Multivariate analysis was performed to verify the effects of demographic and immunization variables on the T2/T0 ratio (expressed as the natural logarithm) of lymphocytes, total serum proteins and IgG, using the R software (The R Foundation for Statistical Computing).

3. Results

Group 1 consisted of 172 subjects, recruited in military schools, (mean age $\pm$ standard deviation 20.7 ± 2.8 ; males 145) and group 2 of 89 members of the Regiment "Lancieri di Montebello" (located in Rome), actively engaged in a nine-month-long international mission to Lebanon (mean age $\pm$ standard deviation 30.9 ± 4.6 ; males 84). Mean age and male/female rate were significantly different between the two groups ($p < 0.0001$ and $p = 0.31$, respectively). Administered vaccines were the following: alum-adsorbed tetanus/diphtheria administered to 44 (26%) group 1 and 21 (24%) group 2 subjects, inactivated polio administered to 57 (33%) group 1 and 18 (20%) group 2 subjects, measles/mumps/rubella administered to 54 (31%) group 1 and 1 (1%) group 2 subjects, varicella administered to 8 (5%) group 1 and 1 (1%) group 2 subjects, polysaccharide tetravalent (A, C, W₁₃₅, Y) meningococcal meningitis administered to 167 (97%) group 1 and 73 (82%) group 2 subjects, virosome-adsorbed hepatitis A administered to 56 (32%) group 1 and 7 (8%) group 2 subjects, alum-adsorbed hepatitis B administered to only 4 (4%) group 2 subjects, MF59-adsorbed influenza administered to 2 (1%) group 1 and 89 (100%) group 2 subjects, and oral typhoid administered to 2 (1%) group 1 and 64 (72%) group 2 subjects. The number of co-administered vaccines in both groups was: 51 (30%) group 1 subjects received only one vaccine, 48 (28%) group 1 and 16 (18%) group 2 subjects received 2 vaccines, 55 (32%) group 1 and 48 (54%) group 2 subjects received 3 vaccines, 16 (9%) group 1 and 22 (25%) group 2 received 4 vaccines whereas 2 (1%) group 1 and 3 (3%) group 2 subjects received 5 vaccines.

3.1. Adverse events

No clinical post-vaccine serious adverse event was reported. One out of 172 group 1 subjects (0.6%), who had received meningococcal vaccination, reported headache, whereas one out of 55 (2%), who had received MMR vaccination, reported a reaction at the site of inoculation. Ten out of 89 group 2 subjects (11%), who had received influenza vaccination, reported small local and systemic side effects. In particular, fever (>37.5 °C, 3 subjects), fever accompanied by general malaise and fatigue (4 subjects), malaise, asthenia and local reactions (1 subject), headache and arthralgia (1 subject) and headache and local reactions (1 subject) were recorded. Influenza appeared the most reactogenic vaccine,

Table 1Mean values $\pm$ SD of peripheral blood cell counts, serum protein and immunoglobulin measures at three time points in group 1 and 2 subjects.

	T0 mean value $\pm$ SD	T1 mean value $\pm$ SD	T2 mean value $\pm$ SD	p
Group 1				
Leukocytes (cells/ μ l)	5950 $\pm$ 1500	6080 $\pm$ 1420	6360 $\pm$ 1710	NS ^a
Neutrophils (cells/ μ l)	3659 $\pm$ 1232	3439 $\pm$ 1254	3763 $\pm$ 1513	NS
Lymphocytes (cells/ μ l)	1734 $\pm$ 470	1902 $\pm$ 465	2006 $\pm$ 452	T0 vs T1 < 0.01; T0 vs T2 < 0.0001
Total proteins (g/dl)	7.58 $\pm$ 0.46	7.47 $\pm$ 0.49	7.73 $\pm$ 0.43	T0 vs T2 < 0.01; T1 vs T2 < 0.001
Alpha 1 globulin (g/dl)	0.32 $\pm$ 0.03	0.32 $\pm$ 0.03	0.30 $\pm$ 0.03	NS
Alpha 2 globulin (g/dl)	0.73 $\pm$ 0.1	0.75 $\pm$ 0.1	0.75 $\pm$ 0.1	NS
Beta globulin (g/dl)	0.80 $\pm$ 0.1	0.80 $\pm$ 0.1	0.81 $\pm$ 0.08	NS
Gamma globulin (g/dl)	1.21 $\pm$ 0.19	1.23 $\pm$ 0.2	1.26 $\pm$ 0.19	NS
IgG (mg/dl)	1132 $\pm$ 189	1188 $\pm$ 196	1186 $\pm$ 195	T0 vs T1 and T0 vs T2 < 0.01
IgM (mg/dl)	112 $\pm$ 46	123 $\pm$ 48	121 $\pm$ 49	NS
IgA (mg/dl)	191 $\pm$ 85	199 $\pm$ 80	202 $\pm$ 83	NS
Group 2				
Leukocytes (cells/ μ l)	6506 $\pm$ 1783		6972 $\pm$ 1527	NS
Neutrophils (cells/ μ l)	3599 $\pm$ 1414		3953 $\pm$ 1282	NS
Lymphocytes (cells/ μ l)	2289 $\pm$ 542		2166 $\pm$ 603	NS
Total proteins (g/dl)	6.3 $\pm$ 0.4		6.3 $\pm$ 0.4	NS
Alpha 1 globulin (g/dl)	0.24 $\pm$ 0.08		0.23 $\pm$ 0.02	NS
Alpha 2 globulin (g/dl)	0.33 $\pm$ 0.1		0.35 $\pm$ 0.08	NS
Beta globulin (g/dl)	0.65 $\pm$ 0.1		0.67 $\pm$ 0.1	NS
Gamma globulin (g/dl)	0.97 $\pm$ 0.2		1.06 $\pm$ 0.2	NS
IgG (mg/dl)	1131 $\pm$ 314		1131 $\pm$ 367	NS
IgM (mg/dl)	92 $\pm$ 31		99 $\pm$ 42	NS
IgA (mg/dl)	219 $\pm$ 83		231 $\pm$ 88	NS

^a NS = not significant.

particularly in relation to malaise and fever, which were present, isolated or together, in 8/89 (9%) subjects.

3.2. Blood parameters

Results (mean values $\pm$ standard deviation) of the evaluation of the peripheral blood cell counts, serum protein electrophoresis and serum Ig in the group 1 are shown in Table 1. To note, peripheral blood lymphocytes were significantly increased from T0 to T1 and T2 ($p < 0.01$ and $p < 0.0001$, respectively). Moreover, a significant increase of the serum total proteins was observed from T0 and T1 to T2 ($p < 0.01$ and $p < 0.001$, respectively), as well as of serum IgG from T0 to T1 and T2 ($p < 0.01$). No monoclonal peak was observed at electrophoresis.

Results (mean values $\pm$ standard deviation) of the evaluation of the peripheral blood cell counts, serum protein electrophoresis and serum Ig in group 2 are shown in Table 1. No significant increase of blood cells, proteins or IgG, nor monoclonal peaks, were noticed.

The significant increase of circulating lymphocytes, total serum proteins and IgG at T2 compared with T0 was observed only in group 1 and only in the univariate analysis (Table 1). When adjusting for demographic data or immunization variables, no statistically significant differences were observed between groups, excepting the alum-adjuvanted tetanus/diphtheria vaccine, which could influence the increase of circulating lymphocytes, as shown by the multivariate analysis (Table 2).

3.3. Autoantibodies

In group 1 14/172 subjects (8.1%) at T0, 17/172 (9.9%) at T1 and only 4/172 (2.3%) at T2 (T2 vs T0 $p < 0.05$; T2 vs T1 $p < 0.01$) were autoantibody-positive. At T0, 5 subjects were ANA-positive, 1 anti-dsDNA, 3 anti-ENA and 5 APL; at T1, 8 subjects were ANA-positive, 3 anti-ENA, 4 APL and 2 RF. At T2, 3 subjects were ANA-positive (7 subjects turned negative, 1 remained positive and 2 turned positive) and 1 subject was APL-positive (already positive at T0, 3 subjects turned negative). Anti-ENA-, anti-dsDNA- and RF-positive subjects turned negative. None had ANCA.

In group 2, 6/70 (8.5%) pre-vaccination sera samples were autoantibody-positive (in fact, 19 subjects have not been explored for lack of biological material) and these same figures (6/70 [8.5%]) were maintained

at T2. The difference at T2 between the percentage of group 1 and 2 autoantibody-positive subjects was not significant ($p = 0.063$). At T0, 5 subjects had ANA and 1 anti-ENA, and the same was at T2 (among the ANA-positive, 4 maintained the positivity, 1 turned negative, and 1 turned positive).

Considering the two groups together, the baseline autoantibody-positivity was 20/242 (8.3%), whereas at T2 it was 10/242 (4.1%, $p < 0.0000001$). No correlation between number or type of vaccination and autoantibodies was found.

3.4. Microarray analysis

To deeper investigate the presence of self-antigens recognized by antibodies potentially induced by vaccinations, 12 group 1 subjects, who had received ≥ 4 concurrent vaccinations, were studied by a microarray analysis. The protein microarray consists of a library of 1667 human proteins predicted to be secreted and/or trans-membrane. The mean fluorescence intensity (MFI) signals, generated from sera of the study subjects, were statistically analyzed. No significant difference has been observed in the autoantibody recognition between T0 and T1, whereas 10 (0.6%) proteins resulted significant between T0 and T2

Table 2

Multivariate analysis of demographic and immunization variables on lymphocytes, total proteins and IgG levels in the two groups of military.

	Lymphocytes(°)		Total proteins(°)		IgG(°)	
	b-Coefficient	p	b-Coefficient	p	b-Coefficient	p
Females (ref males)	−0.090	NS ^a	0.008	NS	−0.044	NS
Age	0.007	NS	0.002	NS	0.004	NS
Group 1 (ref group 2)	0.272	NS	0.094	NS	0.126	NS
Number of vaccines	−0.145	NS	0.025	NS	−0.005	NS
Mencevax	0.201	NS	−0.016	NS	−0.185	NS
Epaxal	0.062	NS	−0.017	NS	−0.010	NS
Imovax polio	0.217	NS	−0.010	NS	0.069	NS
Diftetal	0.249	0.032	−0.016	NS	−0.042	NS
Fluad	0.096	NS	−0.029	NS	0.017	NS
VivotifBerna	0.105	NS	0.059	NS	0.068	NS
Priorix	0.184	NS	−0.033	NS	−0.015	NS

(°) Natural logarithm of the ratio (T2/T0).

^a NS = not significant.

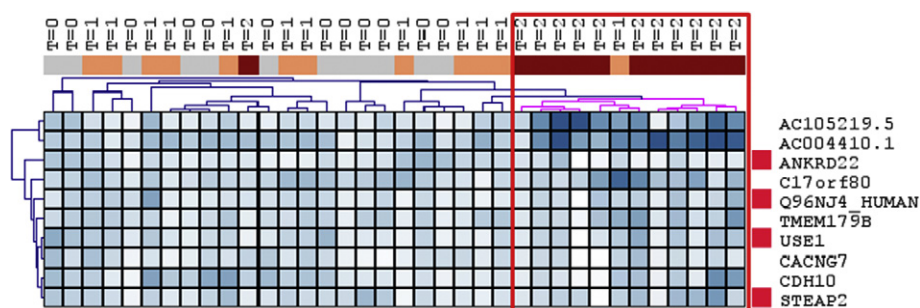


Fig. 1. Heat-map representation of the 10 statistically significant proteins. The individual fluorescence signals on the array of proteins were evaluated for their significance. Ten proteins have been reported as significant by comparing the T0 and T2.

(Fig. 1). However, on the basis of a T2/T0 threshold > 1.5 , the significant proteins were reduced to 4 (Table 3). A further evaluation, based on the comparison between proteins expressed $< 20\%$ at T0 and proteins expressed $> 50\%$ at T2, identified only 3 proteins (Table 4). Finally, number of reactive antigens at each time point with a cut-off > 200 has even been evaluated, without observing any statistically significant difference (Fig. 2).

3.5. HLA typing

The observed MHC gene frequencies were those of the general population [36]. No correlation between the genetic asset and the increase of cells/proteins could be observed (data not shown).

3.6. Cellular response to apoptotic self-epitopes

The analysis of cellular response to apoptotic self-epitopes was restricted to a representative sample of HLA-A2 subjects. In particular, 29 out of 87 evaluated group 1 subjects were HLA-A2. Of these, 11 had received ≥ 4 vaccinations, whereas 18 had received ≤ 3 vaccinations. All the 29 subjects were analyzed for the CD8⁺ T-cell response to apoptotic self-epitopes at T0 and T1 and compared with healthy donors for interferon (IFN)- γ spot forming cells, as detected by ELISPOT assay. No difference has been observed between vaccinated subjects and healthy donors, nor between subjects who had received ≥ 4 and ≤ 3 vaccinations (Fig. 3). Finally, in both categories of vaccinated subjects the number of IFN- γ spot forming cells at T1 was lower than at T0.

4. Discussion

The results obtained in this study show that multiple concurrent immunizations of young adults are safe and not associated to serious post-vaccination clinical and/or laboratory adverse events. Autoantibodies were occasionally detected in some individuals, but their kinetics of appearance and disappearance, including their marked reduction at T2, is in line with the interpretation of a transient and innocuous phenomenon, lacking any pathogenicity. Autoantibodies were cumulatively observed in 20/242 (8.3%) subjects at T0, a figure in line with the literature [37], and in most cases they were low-titer. Moreover, at T2 only 10/242 (4.1%, T0 vs T2 $p < 0.0000001$), subjects were still

autoantibody-positive, thus enabling to rule out any hypothesis of autoimmune post-vaccine stimulation, rather suggesting a sort of negative immune modulation. Some autoantibodies, like ANA, APL, anti-Ro and anti-La are relatively common in healthy populations, while others, like anti-dsDNA, anti-Sm and anti-RNP are rare [38], in agreement with what has been here observed. Post-vaccine autoantibodies turned positive in 3.3% of all subjects at T1 and T2. In a study on healthy adults the percentage of post-vaccine autoantibody-positive subjects was higher than the one observed in the current study (15% one and 13% six months post-vaccination) [39]. In two group 1 subjects, RF switched positive at T1 to disappear at T2, as already observed following the anti-typoid/tetanus vaccination in the Italian military [40]; this is a probable consequence of vaccine-induced immune-complexes formation [41]. However, in group 1 subjects none of the new-onset autoantibodies at T1 was any longer observed at T2, indicating a transient phenomenon. In fact, from the literature cases vaccination-induced autoantibodies appear “benign” and only rarely associated with the expression of autoimmune diseases [40,42].

Moreover, an innovative and validated molecular and immunological approach was applied to 12(7%) group 1 subjects exposed to ≥ 4 concurrent vaccinations; it enabled to exclude a post-vaccine increment of self-antigens recognized by specific murine antibodies. Though this population sample is small, it represents 3/4 of subjects who received at least 4 vaccinations, thus allowing to conclude that multiple vaccinations are likely not associated with autoantibody onset in the nine-month follow-up. In addition, no evidence of immunization-related CD8⁺ T cell response to apoptotic self-epitopes could be observed in 17% group1subjects, representative of both, individuals receiving ≥ 4 (11/18, 61%) and ≤ 3 (18/154, 12%) vaccinations. This result, obtained through a validated new study approach, contributes to downgrade the hypothesis of a possible vaccine-induced cell-mediated autoimmunity. In fact, in autoimmune conditions CD8⁺ T cell response to apoptotic self-epitopes resulted markedly increased [43,44]. Thus, even though anecdotal episodes of post-vaccine autoimmunity onset have been observed, this study is in line with previous reports demonstrating lack of association between systematic autoimmunity onset and vaccine administration [24,45].

A tight reciprocal association between autoimmunity and lymphoproliferation has been observed [15]. Specifically, vaccine/adjuvant-induced autoimmunity could represent a transition phase to lymphoproliferation,

Table 3

Four antigens having a T2/T0 ratio > 1.5 are shown.

Gene symbol	Description	T2	T0	Value fold T2/T0
ANKRD22	Ankyrin repeat domain-containing protein22	194.08	69.84	2,78
Q96NJ4_HUMAN	Putative uncharacterized protein	159.75	105.58	1,51
USE1	Vesicle transport protein USE1	197.0	101.84	1,93
STEAP2	Metalloreductase STEAP2	203.25	121.58	1,67

Table 4

Three percent recognition of antigens selected considering only the T0 $< 20\%$ and T2 $> 50\%$ values.

Gene symbol	Description	T2	T0	Value fold T2/T0
MFSD11	UNC93-like protein MFSD11	2/12 (17)	5/12 (42)	8/12 (67)
CTAGE1	Protein CTAGE-2	2/12 (17)	7/12 (58)	7/12 (58)
PLB1	Phospholipase B1, membrane-ass. Precursor	2/12 (17)	7/12 (58)	7/12 (58)

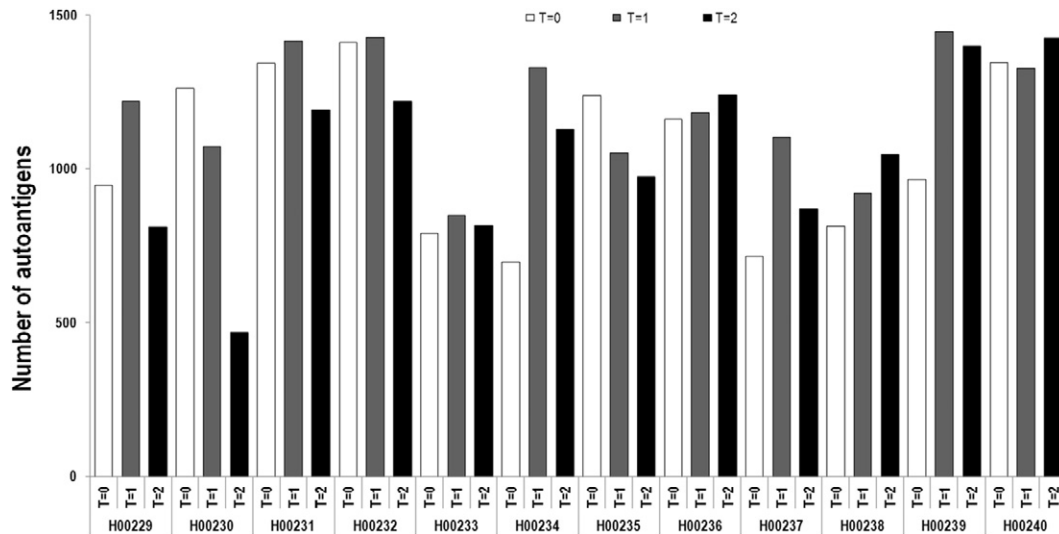


Fig. 2. Autoantigens recognition. Number of antigens recognized by each subject (at T0, T1, and T2, respectively) at a cut-off >200 (of mean fluorescence intensity [MFI] value).

probably as a consequence of long-lasting, scarcely controlled, inflammation. According to this hypothesis, the lack of vaccine-related autoimmunity observed in this study may be considered as further indirect evidence for lack of a pathogenetic link between vaccines and lymphoproliferative disorders. The significant increase in circulating lymphocytes, whole serum proteins and IgG isotype observed in group 1 subjects could be interpreted as a consequence of the physiologic immune stimulation, driven by the administered vaccines/adjuvants. Conversely, a significant increase in circulating lymphocytes, whole serum proteins and IgG isotype was not observed in group 2 subjects. However, when the two groups were jointly considered in a multivariate analysis, there was no difference between the two groups for these parameters and no influence of demographic or immunization intergroup unbalances. In recipients of alum-adsorbed tetanus/diphtheria vaccine a significant increase of circulating lymphocytes was observed. This phenomenon is likely a consequence of the alum-driven inflammasome stimulation, inflammatory cytokine secretion and polyclonal activation [46]. However, such increase is not associated with an *in vitro* enhancement of T cell proliferation to vaccines molecule, thus indirectly confirming the interpretation of a polyclonal activation of the immune system instead of a specific response to vaccine as responsible of circulating lymphocyte increase (data not shown, manuscript in preparation). Recently, the role of adjuvants, mainly alum, for induction of inflammation, chronic immune stimulation and polyclonal lymphocyte activation has been underlined [15]. MF59, which is present in the influenza vaccine and was nearly exclusively

administered to group 2 subjects, has, instead, been shown to very selectively stimulate specific immune response, without inducing B-cell polyclonal activation [47,48]. Data here presented, including the lack of post-vaccine polyclonal or monoclonal gammopathy onset, do not support the hypothesis of an association between vaccinations and lymphoproliferative disorders, even though the observation period was relatively short. However, it has to be underlined that the anecdotal prospective cases of post-vaccine lymphoproliferative disorder development generally occurred within few days or months from vaccination [7,10,49]. Moreover, in the literature, multiple vaccinations are not associated with any type of cancer [50], including multiple myeloma, even during a very long follow-up [13].

The strength of this study consists in its prospective nature as well as in the innovative molecular and immunological approaches, never used before in this context, applied on a reduced, although representative, population sample. The main weakness is its relatively short follow-up.

5. Conclusion

The currently administered vaccines to Italian military personnel resulted safe in a representative population sample. However, it should be stressed that, in agreement with the Italian Ministry of Defense Guidelines, special care was spent to administer a personalized vaccine schedule, based on vaccination and infectious diseases history, to avoid unnecessary immunizations. Despite the observed safety, the reported anecdotal vaccine adverse events suggest that surveillance of vaccinated individuals should be routinely performed, in order to detect any possible side effect.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2017.06.001>.

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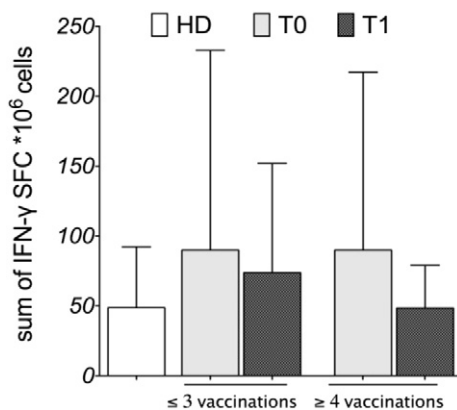


Fig. 3. Detection of autoreactive CD8 + T-cells by ELISPOT assay at T0 and T1: sum of IFN-γ spot forming cells (SFC) for subjects. Comparison between healthy donors and vaccinated subjects at the different time points. Mean values of sum SFC ± SD.

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Supplementary material

Table 1. HLA-A2 binding peptides derived from apoptotic cell-associated proteins.

Organism	Protein	1 st pos [°]	Sequence	Length	Binding capacity (IC50§ nM)					bound
					A*0201	A*0202	A*0203	A*0206	A*6802	
Human	ACTB'	131	AMYVAIQAV	9	2.8	1.5	1.6	7.1	190	5
Human	ACTB	319	ALAPSTMKI	9	4.3	2.9	2.5	40	5.5	5
Human	ACTB	266	FLGMESCGI	9	2.7	71	11	16	3979	4
Human	ACTB	312	RMQKEITAL	9	14	0.29	6.5	28	6667	4
Human	ACTB	348	SLSTFQQMWI	10	5.6	4.5	7.7	791	299	4
Human	ACTB	46	GMGQKDSYV	9	196	17	156	13547	17632	3
Human	ROK [^]	154	SLAGGIIGV	9	0.56	1.0	1.1	0.14	49	5
Human	ROK	67	ALRTDYNASV	10	8.9	5.0	11	349	1354	4
Human	ROK	193	VLIGGKPDRV	10	14	8.5	12	1282	7417	3
Human	ROK	209	ILDLISESPI	10	360	65	1272	2598	3083	2
Human	ROK	122	QLPLESDAV	9	246	601	1270	925	1999	1
Human	LAM1**	496	TIWAANAGV	9	8.9	263	308	22	20	5
Human	LAM1	41	RLAVYIDKV	9	0.16	0.84	0.18	14	1004	4
Human	LAM1	301	SLSSQLSNL	9	1.6	0.57	2.4	104	1377	4
Human	LAM1	361	QLLDVKLAL	9	0.060	98	288	4.0	5848	4
Human	LAM1	291	ELMESRMRI	9	246	1226	284	25188	45	3
Human	LAM1	355	QLNDYEQLL	9	2.0	0.23	18	1493	4808	3
Human	LAM1	388	KLSPSPSSRV	10	50	18	45	563	20979	3
Human	LAM1	488	VLKAGQTVTI	10	263	37	23	4060	15429	3
Human	LAM1	378	KLLEGEERL	10	30	29	3395	1944	20515	2
Human	MYH9"	9	YLYVDKNFI	9	9.3	0.85	4.8	45	217	5
Human	MYH9	108	GLIYTYSGL	9	22	11	10	197	58	5
Human	MYH9	111	YTYSGLFCV	9	0.12	2.1	3.0	0.84	3.2	5
Human	MYH9	145	EMPPHIYAI	9	323	67	110	85	1.8	5
Human	MYH9	186	KVIQYLAYV	9	0.67	0.17	1.2	0.21	1.8	5
Human	MYH9	478	QLFNKHTMFI	9	1.4	0.71	3.2	14	14	5
Human	MYH9	584	WLMKNMDPL	9	9.7	0.36	14	6.0	1.9	5
Human	MYH9	653	QLAKLMATL	9	5.4	1.4	2.9	26	18	5
Human	MYH9	111	YTYSGLFCVV	10	0.21	0.34	0.12	4.2	0.4	5

° = 1st amino acid position; § = IC50 indicates binding affinity, and it is expressed as 50% inhibitory nanomolar concentration, a dash (-) indicates an IC50 >50 000 nM; peptides with IC50 <50 nM are classified as high affinity peptides, peptides with IC50 >50 and <500 nM are classified as intermediate affinity peptides, peptides with IC50 >500 nM are classified as low affinity peptides; ' = actin cytoplasmic 1; ^ = heterogeneous nuclear ribonucleoprotein K; ** = lamin B1; " = non-muscle myosin heavy chain 9.